Structures of quinone-binding sites in *bc* complexes: functional implications

E. A. Berry*, Z. Zhang*†, L.-S. Huang*‡ and S.-H. Kim*‡

*Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720, U.S.A., †Graduate Group of Biophysics, University of California, Berkeley, CA 94720, U.S.A., and ‡Department of Chemistry, University of California, Berkeley, CA 94720, U.S.A.

Overall mechanism

Mitchell's protonmotive Q-cycle (quinone-cycle) mechanism [1,2] for the cytochrome bc_1 complex proposes that ubiquinol is oxidized at a site (Q_o or Q_P) in protonic equilibrium with the P side of the membrane (the side that becomes positive and acidic upon energization), so that the protons released in the reaction go to the P side. One of the electrons reduces the high-potential chain (the Rieske iron–sulphur cluster, cytochrome c_1, and cytochrome c, which is re-oxidized by cytochrome

Abbreviations used: Q cycle, quinone cycle; N side, P side: sides of energy-transducing biological membranes that become, respectively, negative and alkaline or positive and acidic upon energization; MOA, methoxy-acrylate.

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oxidase), providing the driving force for the reaction. The other electron crosses the membrane against (and contributing to) the membrane potential via the two haems of cytochrome *b*, and reduces quinone at another site (Q_i or Q_N). This site is in protonic equilibrium with the N side (the side that becomes negative and alkaline upon energization), so that the protons consumed in the reaction come from the N side. Because only one electron comes to this site for each turnover of the Q_o site, two turnovers are necessary to fully reduce one quinone. Two turnovers of the Q_o site result in net oxidation of one ubiquinol, with two electrons passed on to oxygen, two protons taken up from the N side, and four protons (including the two 'scalar' protons) released to the P side.

Hydrogen-bonding blurs the distinction between protonated and deprotonated states

In considering mechanisms of quinone oxidation or reduction catalysed by enzymes, it is usually assumed that ubiquinone could exist in one of nine discrete states generated by two steps of one-electron reduction and two steps of protonation, occurring in any order:

Q	QH^+	QH_2^{2+}
$Q^{\bullet-}$	$Q^{\bullet}$	$QH_2^{+\bullet}$
Q^{2-}	QH^-	QH_2

If we consider that the carbonyl oxygens of quinone are likely to be involved in hydrogen bonds when the quinone is bound, the protonation state can vary continuously from unprotonated to H-bond accepting and H-bond donating to protonated with no H-bond:

1. N⋮ :O=Φ
2. N⋮•••O=Φ
3. N⋮••H••O=Φ
4. N⋮•••HO-Φ
5. N: HO-Φ

Here, Φ is the phenyl ring of ubiquinone; : is a lone pair of electrons, and =, – and ••• represent double, single and hydrogen bonds respectively. It seems likely that this 'variable state of protonation' is used by enzymes to catalyse oxidation or reduction of quinones. Since the midpoint potential for oxidation of QH_2 to $QH_2^{+\bullet}$ in ethanol/water mixtures is very high, it has been said that deprotonation must precede oxidation. On the other hand, the pK_a for deprotonating QH_2 t QH^- is 12.3, which could make the reaction ver slow at neutral pH. However, if the proton i involved in a hydrogen bond with a potenti proton acceptor, the electron distribution coul vary as the proton moves, effectively going di agonally from QH_2 to $QH^{\bullet}$ and avoiding the ener getically unfavourable states $QH_2^{+\bullet}$ and QH^-.

Hydrogen-bonding can separate uptake or release of protons in the solvent from covalent protonation state of the quinone

The pH dependence of the apparent equilibrium constants for a reaction step indicates whethe protons are involved, and how many. Suppos quinone binds as an H-bond acceptor and quino dissociates as an H-bond donor, taking the proton with it. Then proton uptake will seem to occur a the dissociation step or the binding step (depend ing on the ionization states of the H-bondin residues in the empty site) and not at the electron transfer steps. Thus the pH dependence may no tell where a particular intermediate resides in th continuum between fully deprotonated and fully protonated.

Ubiquinone reduction (Q_N or Q_i) site

Because quinone is reduced at the Q_i site by two sequential one-electron reductions, a semiquinone exists during turnover, and the EPR signal of the semiquinone radical has been observed at relatively high occupancy. Potentiometric titrations monitored by the EPR signal demonstrate that the semiquinone is relatively stable with respect to fully oxidized or reduced quinone, i.e. the two one-electron reductions have roughly the same midpoint potential. The midpoint potential for adding the first electron is pH-independent near neutrality, whereas that for the second electron varies by 120 mV/pH unit [3], suggesting the pathway $Q \rightarrow Q^{\bullet-} \rightarrow QH_2$, with both protons added concomitantly with the second electron. An effect of the redox state of the quinone on the midpoint potential of the high-potential haem of cytochrome *b* has been proposed [4,5] to explain the ≈ 150-mV component in the redox titration of this cytochrome. Spin coupling between the semiquinone and the unpaired electron of cytochrome *b* has been proposed to account for differences between the behaviour of the EPR and visible-absorption signals of cytochrome *b* during redox titrations [6]. Proton Matrix electron-nuclear double resonance (ENDOR) spectroscopy

has indicated deuterium-exchangeable proton(s) strongly coupled to the semiquinone radical [7].

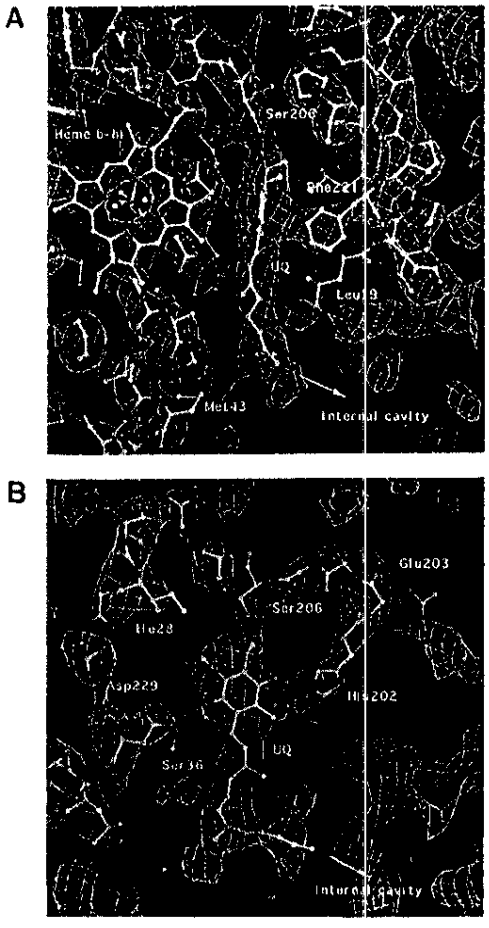
What can the X-ray structures tell us about the mechanism of the reaction at the Q_i site? With the bovine enzyme in the tetragonal space group, indirect evidence for the location of the quinone at the Q_i site was obtained from decreases in density in crystals containing antimycin [8]. A model for ubiquinone was built into a negative-contoured difference density map between an antimycin-containing and an untreated crystal [9].

In maps of the avian protein made using experimental phases, a model was built for antimycin ([10], PDB entry 3BCC) but the density was too tenuous in the untreated crystals to model ubiquinol. However, when phases from the refined structure lacking quinone were used, clear density was obtained for quinone at the Q_i site (Figure 1). A model was built and refined against the data, and is included in the PDB entry 1BCC, upon which the structural description presented here is based. It must be realized, however, that the low resolution of the data and the disorder of the ubiquinone make it impossible to orient the quinone molecule unambiguously, and an alternate orientation in which the head group is rotated 180° is also possible.

Figure 1

Electron density at the Q_i site in native chicken crystals

The refined model of the complex, superimposed on a 2Fo-Fc map, where Fc and Φc are calculated from the refined model with ubiquinone and all atoms within 3.5 Å of the quinone omitted to avoid phase bias, contoured at 0.9 σ. The superimposed model is the refined structure of the complex, including ubiquinone with its headgroup in the centre of each panel. (A) and (B) show two different views related by a 90 ° rotation about a vertical axis.



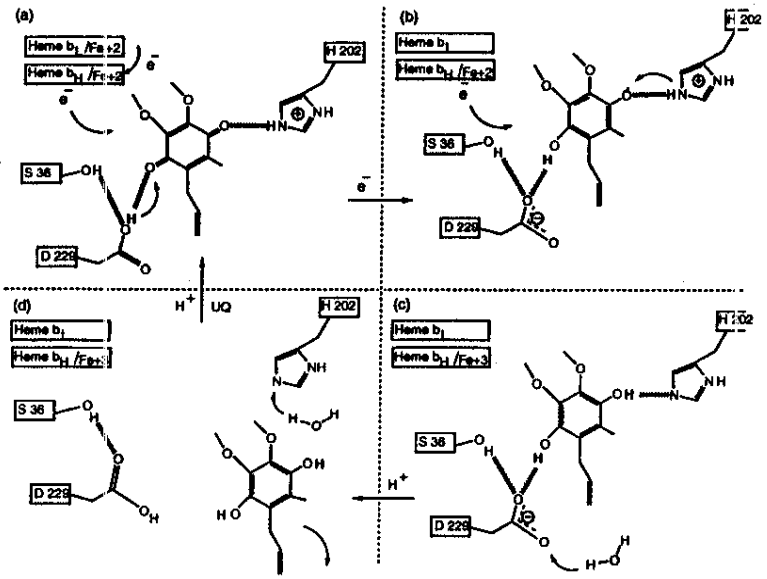
According to the model of entry 1BCC, S-206 in the d-e linker makes an H-bond with the 3-methoxy oxygen of ubiquinone (where 'd-e' refers to a polypeptide stretch linking transmembrane helices D and E of cytochrome *b*; the eight transmembrane helices are designated A–H). The carbonyl oxygen on C_4 H-bonds with H-202 whereas that on C_1 is in a position to H-bond with either or both of D-229 and S-36. When cytochrome *b* and b_6 sequences are aligned, H-202 and D-229 are conserved in mitochondrial and bacterial cytochrome *b*, but not in cytochrome b_6. S-206 becomes N or Q in bacterial cytochromes *b*, but these residues may still have an H-bonding role with different geometry. S-36 is not well conserved, so its H-bonding side-chain cannot be essential for the reaction.

Mutations in the residues corresponding to H-202, D-229 or S-206 result in perturbations of the Q_i-site function or changes in sensitivity to Q_i-site inhibitors (tabulated in [11]). Mutation of H-202 to arginine increased the stability of the semiquinone, whereas mutation to alanine or glutamate eliminated the semiquinone and inactivated the complex, leading to the proposal of H-202 as a quinone ligand [12]. In the model of PDB entry 1BCC, the Nε atom of H-202 is H-bonded to a carbonyl of the quinone, whereas the Nδ atom is exposed to the aqueous phase on the N side of the membrane. The cavity of the Q_i site can be accessed from the bulk lipid phase by a channel through which the tail of the quinone extends into the lipid. It can be accessed from the aqueous phase on the N side by a narrow channel bounded by residues H-202, P-23 and G-205. One possible scheme for the mechanism is the following: water enters the empty site and protonates the residues (D-229 and H-202) involved in binding the quinone. Then, quinone binds and is reduced by sequential one-electron transfers, with the protons moving towards the quinone and away from the protein residues, perhaps compensated in the case

Figure 2

A plausible model for H^+ and e^- transfer details for the reactions at the Q_i site

The protons consumed in reduction of the quinol come from the H-bonding residues D-229 and H-202, which are reprotonated after dissociation of the quinol.



of H-202 by protonation of the delta nitrogen from the aqueous medium. Such a scheme is depicted in Figure 2.

The ubiquinol oxidizing (Q_o or Q_P) site

Q_o site structure

At the time of writing, there have been no reports of visualization of the quinone at the Q_o site. Failure to observe the quinone at the Q_o site may be due to the low content of ubiquinone in the preparations used for crystallization, and the apparently greater affinity of the Q_i site. In any case, we can only speculate on binding modes of ubiquinone based on the binding of Q_o-site inhibitors that have been visualized in the crystals. The description here is based on stigmatellin (PDB entry 3BCC) and as-yet-unsubmitted structures for myxothiazol (structure given in [12a]) and several strobilurin analogues at the Q_o site.

The Q_o site is sandwiched between four strands of protein forming two $\approx 45°$ elbows: the C transmembrane helix with the CD surface helix and the PEWY descending loop of the e-f linker with the EF surface helix (here, the surface helices CD and EF are designated as such since they occur within polypeptide linkers c-d and e-f respectively). The bridging ring of strobilurins, the side-chain methoxy group of myxothiazol and the ring of stigmatellin all make hydrophobic contact with the ring of P-271. The side-chain ring of strobilurins and the first thiazo ring of myxothiazol stack with F-275, which the tail of stigmatellin also touches. However, the rest of the protein contacts are quite different between stigmatellin on one hand and the methoxy-acrylate (MOA) inhibitors [12a] on the other.

Whereas the ring of stigmatellin reaches towards the cluster ligand H-161 of the Rieske protein, the carbonyl end of the pharmacophore of the MOA inhibitors reaches in the opposite direction, towards the haem, through the crack between PEWY and the elbow of helices C and CD. This results in stigmatellin being within hydrogen-bonding distance of the Rieske protein, whereas the MOA inhibitors reach much closer (5 Å) to the haem.

For stigmatellin, the side of the ring away from the Rieske protein probably H-bonds with the carboxylates of E-272, which seems to adopt a different configuration in the presence of stigmatellin. If we consider that stigmatellin is a quinone analogue, this suggests that E-275 may be the ligand for its second carbonyl, and the movement may provide a mechanism for transport of the proton out of the pocket to a hydrophilic region surrounding the haem propionates, from which the proton escapes to the aqueous medium on the P side of the membrane. For the MOA inhibitors, the carbonyl end of the pharmacophore may H-

bond with the backbone N of E-272 and/or with the phenolic OH of Y-274. The other end of the pharmacophore may H-bond with backbone atoms of G-143 and A-144.

The bifurcated reaction at Q_o

From a teleological point of view, bifurcation of the electron pathway at the Q_o site is necessary for efficient energy conservation. From an experimental point of view, the bifurcated reaction is required to explain the inhibition of electron transport by Q_i-site inhibitors such as antimycin. Any mechanism proposed to enforce the bifurcated reaction must explain the inhibition by antimycin as well as the proton-pumping stoichiometry under normal turnover. Four mechanisms will be considered.

In the thermodynamic explanation [13], no special mechanism is required to enforce the bifurcation. It is proposed that the instability of the semiquinone, together with the fact that the Rieske protein can only accept one electron at a time, will prevent both electrons from going down the high-potential chain. The equilibrium constant for the oxidation of ubiquinol by the Rieske protein is so unfavourable that it only proceeds at a competent rate if the semiquinone can immediately reduce cytochrome *b*. If the second electron cannot be transferred to cytochrome *b*, for example because antimycin is present and cytochrome *b* is fully reduced, the reversal of the first electron transfer would be so fast that the Rieske protein could never reduce cytochrome c_1. The necessity for domain movement of the Rieske protein to reduce cytochrome c_1 makes this argument stronger, as the iron–sulphur protein cannot be docked to both the Q_i site and cytochrome c_1 simultaneously, allowing the first electron to 'hop' over Rieske on to c_1, leaving Rieske immediately available to receive the second electron.

Because the lifetime of the semiquinone is exceedingly short, it is unlikely that the semiquinone moves. Thus there is only one quinone-binding site for reduction of both the iron–sulphur protein and cytochrome *b*, and the different positions of the two classes of Q_o-site inhibitors should not be taken to imply two binding modes for ubiquinone. Assuming the ubiquinone ring is near the position of bound stigmatellin and hydrogen-bonded to the Rieske protein when the first electron is transferred to that protein, then it should be in the same position when the second electron is transferred to cytochrome *b*. The distance from the carbonyl oxygen on the first ring of stigmatellin (O12 in structure 3BCC) to the methyl carbon of pyrrole ring A of the low-potential haem is 10.1 Å, and to C_3 of the same ring is 11.1 Å. Application of Dutton and colleague's ruler [14] to the latter distance, with a reorganization energy Lambda between 0.7 and 1.0 and ΔG of 0, predicts a tunneling rate of $(0.2–1.5) \times 10^6$ s^{-1}. For a highly unstable semiquinone, ΔG will be quite negative, further accelerating the reaction. If we want to take into account the pathways available, the highly conserved aromatic Y-132 lies between stigmatellin and the haem. The rate is thus considerably faster than the turnover of the Q_o site following flash activation (around 2000 s^{-1} [15]), so the distance from the stigmatellin site to the low-potential cytochrome *b* is not incompatible with concerted or nearly concerted reduction of the Rieske cluster and cytochrome *b* by ubiquinone in this position. Figure 3 shows possible H-bond details for such a mechanism.

Unfortunately, some additional *ad hoc* assumptions need to be introduced to account for inhibition by antimycin, as can be seen from the following argument. If the semiquinone is a strong-enough oxidant to immediately reoxidize the iron–sulphur protein when cytochrome *b* is reduced, and it is close enough to the low-potential haem of cytochrome *b* to reduce it when cytochrome *b* is oxidized, then what is to prevent the semiquinone from oxidizing cytochrome *b* under conditions of oxidant-induced reduction? Such a reaction has been proposed to account for initiation of the Q cycle under highly reducing conditions. Then respiration could proceed at nearly the normal rate in the presence of antimycin by alternating cycles of (i) an aberrant reaction in which ubiquinone reduces the iron–sulphur protein and the resulting semiquinone oxidizes cytochrome *b*, and (ii) the normal Q-cycle mechanism, which is possible now that the low-potential haem of cytochrome *b* is oxidized.

The second model [16,17] involves a 'catalytic switch' mechanism to prevent inappropriate reduction of the Rieske protein by a semiquinone at Q_o. Just as the camshaft of an automobile engine opens the intake and exhaust valves at specific times in the engine cycle, so electrons from the Q_o site could be alternately gated on to the Rieske protein and cytochrome *b* by some catalytic switch. A conformational change was suggested, and the antimycin- or redox-induced conformational change proposed by Rieske [18] was cited. Arrival of the second electron on the high-potential

cytochrome *b* haem or on quinone at the Q_i site could be the trigger gating the Q_o site to Rieske for the next turnover. Although the mechanism was proposed before any X-ray structure was available, the movement of the Rieske protein could obviously be involved in the gating, as proposed [19]. If the oxidized Rieske protein is not allowed to approach the Q_o site until the second electron has gone to cytochrome *b*, the bifurcated reaction will be ensured. Whereas this is still an attractive hypothesis, there is no evidence to date from the structures for a significant conformational change of the transmembrane helices linked to antimycin binding or redox state, and the flexibility of the 'neck' region of the Rieske protein makes it hard to imagine any way of transmitting a conformational change to the Rieske protein to control the movement of its head.

The third mechanism is based on the inference of two different binding modes for quinone species at Q_o from the different binding positions of the two classes of Q_o-site inhibitor. One end of the pharmacophore of the MOA inhibitors is in van der Waals contact with the ring of Y-132, which is nearly in contact with the low-potential haem. At the same time, these inhibitors do not approach the iron–sulphur protein closely enough to form a hydrogen bond. Thus it has been proposed [20] that after transfer of the first electron to the iron–sulphur protein, the hydrogen bond breaks and the semiquinone flips into the position of the MOA inhibitors before reducing cytochrome *b*. This second binding site could be much more favourable for the semiquinone so that, if cytochrome *b* cannot accept the electron, the semiquinone rarely visits its first binding site and thus transfer of the second electron to the Rieske protein is slowed greatly. This model predicts high occupancy of a semiquinone under conditions of oxidant-induced reduction, and the failure to observe the radical by EPR spectroscopy under these conditions [13] needs to be explained.

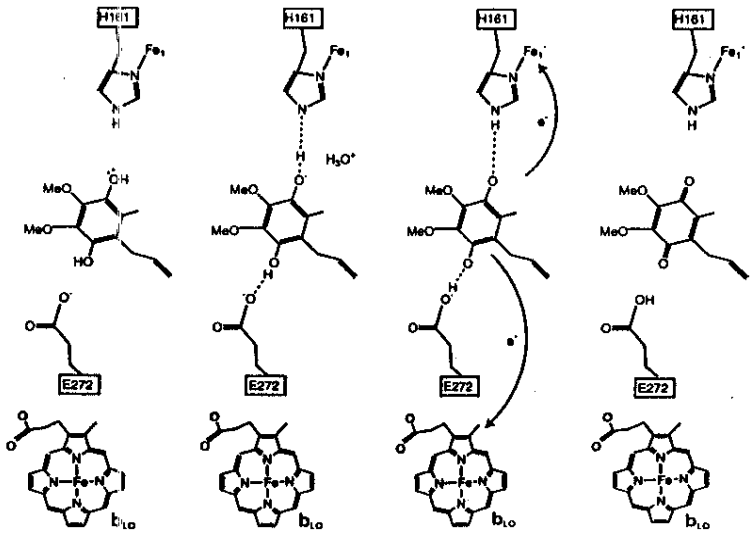
The fourth mechanism to be considered is the proton-gated affinity-change mechanism [21]. In this mechanism the semiquinone formed upon transfer of the first electron to the Rieske protein, like stigmatellin, binds tightly to the Rieske protein and raises its midpoint potential above that of cytochrome c_1. The Rieske protein is thus blocked, both physically and thermodynamically, from disposing of the first electron until after deprotonation and transfer of the second electron converts the semiquinone into the weakly binding species ubiquinone.

This model also predicts accumulation of the semiquinone under conditions of oxidant-induced reduction, but the failure to observe the semiquinone can be explained by assuming that the radical is spin-coupled with the unpaired electron of the reduced Rieske protein. It also predicts that the Rieske protein is reduced under these conditions, which could not be detected by the EPR

Figure 3

A plausible model for H^+ and e^- transfer details for the reactions at the Q_o site

Hydroquinone binds to deprotonated E-272 of cytochrome *b* and binds with release of a proton from H-161 of the Reiske protein. After electron transfer the oxidized quinone unbinds, leaving E-272 and H-161 protonated.



signal but which might be determined from visible CD spectra. Like the first model, it requires that the second electron be transferred from semiquinone in the stigmatellin position to the low-potential haem of cytochrome *b* and, as discussed above, this seems feasible.

Movement of the Rieske protein head domain

Comparison of the structures obtained from different crystal forms shows that the extrinsic domain of the Rieske protein can occupy different positions relative to the other subunits. In PDB structures 1QCR and 3BCC, the iron–sulphur cluster is near the Q_o site. In PDB structures 1BCC and 1BE3, the cluster is near the haem of cytochrome c_1. In either position, the cluster is too far from one of its redox partners for electron transfer to occur at the observed rates. This, together with the highly disordered state of the Rieske head domain in some crystals [8], has led to the proposal that the head domain of the Rieske protein moves during the catalytic cycle, ferrying electrons from ubiquinol at the Q_o site to cytochrome c_1 [10]. A third 'intermediate' position has been identified in one monomer of the hexagonal ($P6_5$, PDB entry 1BGY) bovine crystals [22].

We want to consider what factors affect the position of the Rieske protein, and the mechanism by which they do. For the sake of brevity we will use a model here which assumes that the Rieske head domain simply diffuses, within the limits set by its connection to the transmembrane domain, and binds to different sites depending on their relative affinity, ignoring other possibilities. Most of the results to date can be explained with the following assumptions.

If the Q_o site is unoccupied, Rieske binds most strongly to cytochrome c_1 and is found in the cytochrome c_1 position seen in the native chicken structure 1BCC. With stigmatellin in the Q_o site, as in the inhibited chicken structure 3BCC, binding of the reduced Rieske protein at the Q_o site becomes much stronger, presumably because of the hydrogen bond between the Rieske-cluster ligand H-161 and the ring of the inhibitor [10]. With 5-n-undecyl-6-hydroxy-4,7-dioxobenzoquinone (UHDBT) or ubiquinone, the binding at Q_o is stronger than at cytochrome c_1, but by a smaller margin. In the untreated tetragonal bovine crystals [8], Rieske protein is observed in the 'tight' cytochrome *b* position, but with poor electron density. We speculate that this is due to low occupancy as well as disorder, and that this position is observed because the Q_o site is partially occupied by quinone. Inhibitors stigmatellin and UHDBT improve the density [9], because they bind Rieske more tightly and are present at full occupancy. MOA inhibitors displace what quinone is there and cannot themselves bind the Rieske, and result in the cytochrome *c* position or an unbound 'loose' state [9]. Thus far we have not addressed the redox state of Rieske, but it may be that only the reduced state binds to ubiquinone, UHDBT and stigmatellin. In the case of stigmatellin, it has been observed that the inhibitor results in complete reduction of the Rieske, because tight binding to the reduced form effectively raises the midpoint potential of the cluster to the point at which it is reduced by endogenous reducing agents.

In the EPR experiments on oriented membranes of bacterial bc_1 complex [23], the bc_1 preparation has excess ubiquinone [24], so it can be expected that the Q_o site is fully occupied. The same orientation of the chemically reduced Rieske protein is seen with stigmatellin or UHDBT as without inhibitors, and was attributed to the proximal (cytochrome *b*) position. However, the lack of effect of myxothiazol under these conditions cannot be explained with these assumptions. When the membranes are frozen and Rieske is subsequently reduced by gamma irradiation, the g_x vector has a different orientation, presumably the loose or cytochrome c_1 state. This suggests that the oxidized Rieske protein does not bind to quinone at Q_i. When both Rieske and the quinol at the Q_o site were reduced with dithionite, the same (proximal) orientation was seen as when the quinone was oxidized, suggesting that reduced Rieske can bind to either quinol or quinone at the Q_i site. It might have been supposed otherwise, since the line shape and *g* value of the Rieske protein with reduced quinone superficially resemble those with an unoccupied Q_o site, or purified soluble Rieske fragment. Further characterization of the oriented bc_1 system, as well as more crystal structures at defined redox states and quinone occupancy, would be useful to more fully explain the movement of the Rieske protein.

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